Cardiovascular Topics

Melatonin prevents cardioprotection induced by a multi-cycle ischaemic preconditioning protocol in the isolated perfused rat heart

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Summary

The powerful cardioprotective actions of melatonin, the chief secretory product of the pineal gland, have been attributed largely to its free radical-scavenging properties. Free radicals play an important role in the triggering action of ischaemic preconditioning, the phenomenon whereby exposure of the heart to one or more short episodes of ischaemia leads to protection against a subsequent long period of ischaemia. The aim of this study was, therefore, to establish whether melatonin, in view of its free radical-scavenging ability, would affect the beneficial actions of preconditioning.

Isolated, perfused, working hearts were subjected to 1 × 5 minute or 3 × 5 min ischaemic preconditioning protocols, in the presence or absence of melatonin (50 μM), followed by 20 minutes global ischaemia and 30 minutes reperfusion. Use was also made of sodium nitroprusside (100 μM), a nitric oxide (NO) donor and preconditioning mimetic. Using functional recovery as the endpoint, melatonin abolished the cardioprotective effects of a multi-cycle (3 × 5 min) preconditioning protocol, while having no effect on a one-cycle (1 × 5 min) protocol or SNP (1 × 5 or 3 × 5 min) preconditioning.

The results suggest that free radicals play an important role in the cardioprotection induced by a multi-cycle ischaemic preconditioning protocol and that this process could be attenuated by a potent scavenger such as melatonin.

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Melatonin, the chief secretory product of the pineal gland, is a direct scavenger of free radicals and has indirect anti-oxidant effects due to its stimulation of the expression and activity of anti-oxidative enzymes such as glutathione peroxidase.[1,2] The crucial role of free radicals in the pathophysiology of the ischaemic heart is well established. Therefore, it is not surprising that melatonin has been shown to be a powerful cardioprotective agent: melatonin reduces infarct size,[3,4] improves functional recovery of the working rat heart[5,6] and reduces arrhythmias after coronary artery ligation.[7,8] These cardioprotective actions of melatonin have been attributed largely to its free radical-scavenging properties,[5,9,10] although a recent study demonstrated that the melatonin receptor is also involved.[6]

Although it is generally accepted that free radicals can be detrimental to biological tissues, it has recently been discovered that reactive oxygen species (ROS) can function as signaling molecules.[11] In particular, ROS have been shown to play a triggering role in ischaemic preconditioning, the phenomenon whereby exposure of a heart to one or more short episodes of ischaemia leads to protection against a subsequent long period of ischaemia. For example, a preconditioning protocol of 4 × 5 min ischaemia is associated with a significant loss of glutathione, while administration of N-acetylcysteine, an anti-oxidant, blocks the protective effects of preconditioning.[12]

It has recently been shown[13,14] that opening of mitochondrial K_{ATP} channels by P1075 or diazoxide triggers the preconditioning state by generating mitochondrial ROS. Conversely, blocking P1075-induced ROS production by either glibenclamide or 5-hydroxydecanoate completely reverses P1075 anti-infarct effects.[13] The triggering action of ROS was further confirmed by the finding that its generation by compounds such as bradykinin[15] or SNAP[16] mimics ischaemic preconditioning.

In view of the above, the question arose whether melatonin, because of its free radical-scavenging actions, will affect the beneficial actions of ischaemic preconditioning. The aim of the present study was, therefore, to establish whether melatonin: (1) has a detrimental effect on either a one-cycle or multi-cycle ischaemic preconditioning protocol, and (2) will affect the cardioprotective effects of NO, a

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powerful preconditioning mimetic. In view of its cardioprotective effects when present during reperfusion,[6] melatonin was added during the preconditioning protocol only, before the onset of sustained ischaemia.

Materials and methods

Male Wistar rats weighing 220 to 250 g were used in all experiments. Before anaesthesia (30 mg pentobarbital, ip), rats were allowed free access to food and water. The project was approved by the ethical committee of the Faculty of Health Sciences, University of Stellenbosch and conformed to the *Guide for the Care and Use of Laboratory Animals*.[17]

Melatonin and sodium nitroprusside were obtained from Sigma Chemical Co. All other reagents were of Analar grade and obtained from Merck, Cape Town.

Perfusion technique

After removal and arrest in ice-cold saline, the heart was mounted via the aorta onto the aortic cannula and perfused with Krebs-Henseleit buffer (composition in mM: NaCl 119, KCl 4.74, $CaCl_2$ 1.25, $MgSO_4$ 0.6, Na_2SO_4 0.59, KH_2PO_4 1.79, $NaHCO_3$ 24.9, glucose 10) which was oxygenated with 95% O_2, 5% CO_2 at 37°C. Hearts were initially perfused in a non-recirculating manner at 100 cm H_2O to stabilise, followed by atrial perfusion (preload 15 cm H_2O, afterload 100 cm H_2O).

Myocardial temperature was thermostatically controlled by inserting a temperature probe into the pulmonary artery. Global ischaemia was induced by reducing flow to zero. Temperature during global ischaemia was maintained constant at 36.5°C by surrounding the heart with a water-jacket. Aortic and coronary flows were measured manually, while aortic pressure and heart rate were monitored using a Viggo-Spectramed pressure transducer connected to a computer. Total work performance (pressure power + kinetic power) developed by the heart was calculated by the formulae of Kannengieser *et al*.[18] Measurements of coronary and aortic flow rates, heart rate and peak systolic pressure were made before and after ischaemia.

Perfusion protocols (Fig. 1)

- Non-preconditioned hearts (non-PC): hearts were stabilised for 60 min, followed by 20 min global ischaemia and 30 min reperfusion.
- Ischaemic preconditioned hearts (IPC): two groups were studied:
  - 1 × 5 min IPC: hearts were stabilised for 50 min, followed by 5 min global ischaemia and 5 min reperfusion. Hearts were then subjected to ischaemia and reperfusion as described for non-PC hearts.
  - 3 × 5 min IPC: hearts were stabilised for 30 min followed by three episodes of 5 min global ischaemia, interspersed with 5 min reperfusion. Hearts were then subjected to ischaemia and reperfusion as described above.
- Melatonin-treated ischaemic preconditioned hearts: two groups were studied:
  - 1 × 5 min IPC + melatonin: hearts were stabilised for 35 min, followed by administration of melatonin (50 μM) for 5 min, 5 min global ischaemia, 5 min reperfusion in the presence of melatonin (50 μM) and 10 min reperfusion in the absence of melatonin. This was followed by ischaemia and reperfusion as described above.
  - 3 × 5 min IPC + melatonin: hearts were stabilised for 25 min, followed by administration of melatonin (50 μM) for 5 min, three episodes of 5-min global ischaemia, interspersed with 5 min reperfusion in the presence of melatonin (50 μM), followed by a 10-min washout period (in absence of melatonin) before subjecting hearts to 20 min global ischaemia and 30 min reperfusion.
- NO-preconditioned hearts (NO-PC): two groups were studied.
  - 1 × 5 min NO-PC: hearts were stabilised for 50 min, followed by administration of sodium nitroprusside (SNP) (100 μM) for 5 min and 5 min washout. Hearts were then subjected to ischaemia and reperfusion as described for non-PC hearts.
  - 3 × 5 min NO-PC: hearts were stabilised for 30 min,

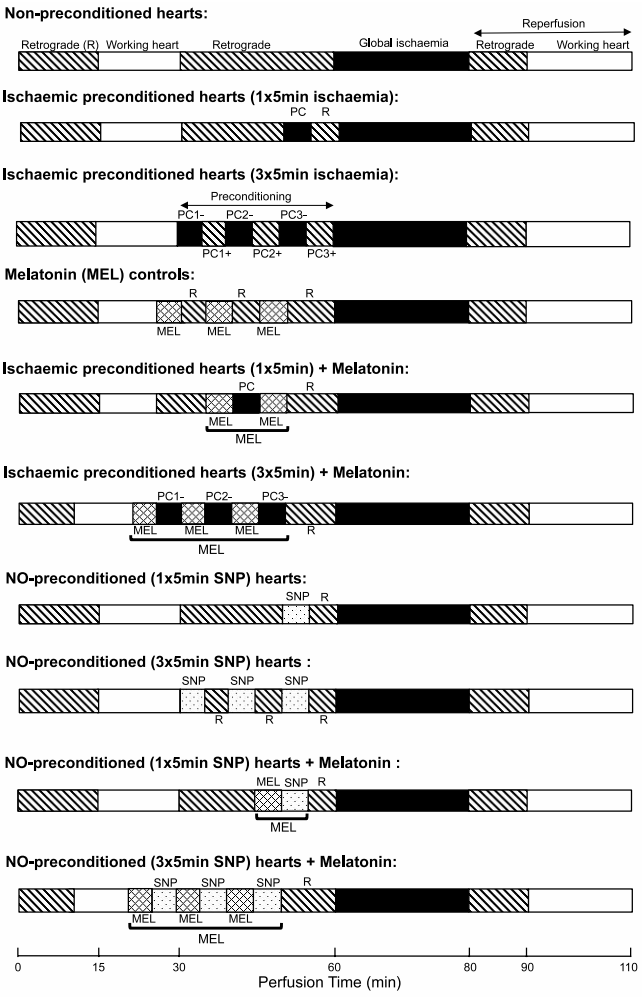

Fig. 1. Perfusion protocols. Abbreviations: PC: preconditioning, R: reperfusion, Mel: melatonin, SNP: sodium nitroprusside.

followed by three 5-min administrations of SNP (100 μM), interspersed by 5 min reperfusion. Hearts were then subjected to ischaemia and reperfusion as described above.

- Melatonin-treated NO-preconditioned hearts: two groups were studied.
  - 1 × 5 min NO-PC + melatonin: hearts were stabilised for 40 min, followed by administration of melatonin (50 μM) for 10 min, the last 5 min of which SNP (100 μM) was added, followed by 5 min washout. Hearts were subjected to ischaemia and reperfusion as described above.
  - 3 × 5 min NO-PC + melatonin: hearts were stabilised for 20 min, followed by 5-min administrations of melatonin (50 μM), followed by three 5-min administrations of SNP (100 μM) and melatonin (50 μM), interspersed by 5-min perfusions in the presence of melatonin alone. Hearts were then subjected to a 10-min washout period, followed by ischaemia and reperfusion as described above.
- Melatonin controls: hearts were stabilised for 25 min. Melatonin (50 μM) was then administered for three 5-min periods, interspersed by 5-min reperfusions without melatonin and followed by a 10-min washout. Hearts were then subjected to 20 min ischaemia and reperfusion as described above.

Statistics

Results are expressed as means (± SEM). Multiple comparisons were analysed by one-way analyses of variance (ANOVA) and the Bonferroni correction was applied as *post hoc* test. *P*-values < 0.05 were considered significant.

Results

Effect of melatonin on ischaemic preconditioning-induced cardioprotection

Non-preconditioned hearts subjected to 20 min global ischaemia showed a significant reduction in aortic flow, cardiac output, PSP, heart rate and total work performed during reperfusion. Melatonin *per se*, when administered for 3 × 5 min followed by 10 min washout before the onset of global ischaemia had no effect on functional recovery during reperfusion and values similar to those of non-preconditioned hearts were obtained. Preconditioning with 1 × 5 min or 3 × 5 min global ischaemia improved functional recovery during reperfusion to a similar extent. For example, aortic output during reperfusion averaged 19.1 ± 2.1 and 19.07 ± 1.6 ml/min, respectively, compared to 6.3 ± 1.7 ml/min in non-preconditioned hearts (a mean increase of 203%). Similarly, total work performance was improved by 76 and 88% by 1 × 5 min and 3 × 5 min preconditioning protocols, respectively (Table I).

Melatonin, when administered before and after 1 × 5 min ischaemic preconditioning had no effect on the beneficial effects of this preconditioning protocol. However, melatonin administered before and during a multi-cycle preconditioning protocol (3 × 5 min), completely abolished the beneficial effect of ischaemic preconditioning and the values obtained were similar to those observed in non-preconditioned hearts. In addition, heart rate was significantly lower in melatonin-treated 3 × 5 min preconditioned hearts than in the corresponding 3 × 5 min preconditioned hearts (Table I).

TABLE I. EFFECT OF MELATONIN (50 μM) ON ISCHAEMIC PRECONDITIONED CARDIOPROTECTION AFTER 20 MIN GLOBAL ISCHAEMIA

	Coronary flow rate (ml/min)	*Aortic output (ml/min)*	*Cardiac output (ml/min)*	*Peak systolic pressure (mmHg)*	*Heart rate (beats/min)*	*Total work (mW)*
Before ischaemia (control values) (58)						
	15.7 ± 0.2	41.6 ± 0.6	57.3 ± 0.7	100.5 ± 0.8	258 ± 4	13.11 ± 0.2
During reperfusion						
Non-preconditioned (13)	11.3 ± 0.8	6.3 ± 1.7	17.5 ± 1.9	77.8 ± 3.5	214 ± 12	3.57 ± 0.54
Non-preconditioned + melatonin (9)	12.8 ± 1.0	7.8 ± 1.8	19.8 ± 2.6	82.6 ± 2.9	222 ± 7	3.71 ± 0.56
Preconditioned (1 × 5 min) (8)	12.8 ± 0.7	19.1 ± 2.1*+	31.9 ± 2.3*+	87.1 ± 1.1	251 ± 9	6.28 ± 0.51*+
Preconditioned (3 × 5 min) (13)	13.2 ± 0.7	19.07 ± 1.6*+	31.2 ± 1.8*+	87.9 ± 1.2	254 ± 9	6.72 ± 0.45*+
Preconditioned (1 × 5 min) + melatonin (7)	13.7 ± 0.6	13.3 ± 3.1	27.0 ± 3.0	86.6 ± 1.5	261 ± 9	5.62 ± 0.54
Preconditioned + (3 × 5 min) melatonin (8)	9.1 ± 2.7	7.1 ± 2.9**	16.2 ± 6.11**	53.5 ± 15.7**+	149 ± 44**	3.11 ± 0.99**

Numbers in parentheses indicate number of hearts
*$p < 0.01$ vs non-preconditioned; +$p < 0.05$ vs non-preconditioned + melatonin;
**$p < 0.01$ vs preconditioned 3 × 5 min.

TABLE II. EFFECT OF MELATONIN (50 μM) ON SNP-INDUCED CARDIOPROTECTION AFTER 20 MIN GLOBAL ISCHAEMIA

	Coronary flow rate (ml/min)	*Aortic output (ml/min)*	*Cardiac output (ml/min)*	*Peak systolic pressure (mmHg)*	*Heart rate (beats/min)*	*Total work (mW)*
Before ischaemia (control values) (39)						
	16.0 ± 0.3	40.7 ± 0.6	56.7 ± 0.8	103.3 ± 1.1	256 ± 5	13.32 ± 0.28
During reperfusion						
Non-preconditioned (13)	11.3 ± 0.8	6.3 ± 1.7	17.5 ± 1.9	77.8 ± 3.5	214 ± 12	3.57 ± 0.54
SNP (1 × 5 min) (7)	13.2 ± 0.7	13.7 ± 3.0	26.9 ± 3.2^{+}	88.7 ± 3.0	245 ± 9	5.44 ± 0.78
SNP (3 × 5 min) (6)	13.6 ± 0.6	14.1 ± 1.3	27.7 ± 1.1^{+}	89.7 ± 1.6	229 ± 13	5.55 ± 0.31^{+}
SNP (1 × 5 min) + melatonin (6)	15.6 ± 0.6*	19.0 ± 2.5*	34.9 ± 2.82*	92.3 ± 3.2^{+}	234 ± 14	7.32 ± 0.87^{+}
SNP (3 × 5 min) + melatonin (7)	15.4 ± 0.4*	15.4 ± 2.4*	30.79 ± 2.74*	91.7 ± 2.2^{+}	235 ± 11	6.37 ± 0.65^{+}

SNP: 100 μM, administered 1 × 5 min or 3 × 5 min. Numbers in parentheses indicate number of hearts
*$p < 0.01$ vs non-preconditioned; $^{+}p < 0.05$ vs non-preconditioned.

Effect of melatonin on SNP-induced cardioprotection

Pharmacological preconditioning with SNP, a NO donor, for 1 × 5 min or 3 × 5 min before the onset of sustained ischaemia also caused a significant improvement in cardiac output (54 and 58% respectively). Total work was also significantly improved (55%) in the case of 3 × 5 min SNP. Interestingly, administration of melatonin before and during preconditioning with SNP further augmented the beneficial effects of the NO donor regardless of the protocol used, although not to a significant extent. However, with both protocols, the coronary flow rate, aortic output, cardiac output, peak systolic pressure and total work were significantly higher in the presence of melatonin, compared with those of non-preconditioned hearts (Table II).

Discussion

To study the effects of melatonin on ischaemic preconditioning, melatonin was used at a pharmacological dose (50 μM). This concentration has been shown to be cardioprotective in the perfused rat heart, while 25 μM was found to be ineffective.[6] Although most workers[5,6] used melatonin at pharmacological doses, it has also been shown to be cardioprotective at physiological doses in *in vivo* studies.[7,8]

The powerful cardioprotective actions of melatonin have been largely attributed to its free radical-scavenging abilities.[5,9,10] However, this particular property of melatonin could also have adverse actions in certain circumstances, in view of the triggering role of free radicals in ischaemic preconditioning. The results obtained in our study show that melatonin does indeed affect the outcome of ischaemic preconditioning, albeit dependent on the protocol used: it had no effect on the outcome of a one-cycle preconditioning protocol, while causing a significant inhibition of the beneficial effects of a three-cycle preconditioning protocol (Table 1).

These results are rather difficult to interpret, since the cardioprotection, using functional recovery during reperfusion as endpoint, was similar in one- and three-cycle preconditioning protocols (Table I). Although a few other studies could also not demonstrate differences between one or more preconditioning cycles in rabbits,[19,20] these findings are in contrast to most other previous studies evaluating the effect of the number of cycles. For example, a multi-cycle preconditioning protocol has been found to be more effective than a one-cycle protocol in rats[21,22] and pigs,[23] using infarct size as endpoint. It is also possible that the endpoint used (infarct size versus functional recovery) played a role in the above discrepancies, since we have previously shown that infarct size is a more sensitive indicator of cardioprotection than functional recovery.[24]

The finding that melatonin is able to block a multi-cycle, but not a single-cycle preconditioning protocol is surprising, in view of other studies where it was found that the latter was more susceptible to pharmacological manipulation than a multi-cycle protocol. It was suggested that when preconditioning is induced with multiple cycles, the cumulative dose of mediators released, and therefore alterations in downstream signal transduction pathways may be much greater than those achieved in a single cycle.[21] Therefore, blockade of a single pathway or mechanism of protection may be insufficient to block protection induced by a multi-cycle protocol.

A possible explanation for the inability of melatonin to block a single-cycle preconditioning protocol is that free radical generation is not an important trigger after only one episode of ischaemia. Our data suggest that free radical generation becomes a more important trigger during a three-cycle protocol. Sandhu and coworkers[21] also suggested that data obtained with pharmacological inhibitors in multi-cycle protocols reveal only those factors that are non-redundant and, therefore, important in the preconditioning process. The significance of free radicals in multi-cycle preconditioning protocols has been demonstrated in several studies (for example, see reference 12).

The ability of melatonin to block ischaemic preconditioning via free radical scavenging was further evaluated using the preconditioning mimetic nitric oxide (NO). Despite

initial controversial results, the role of NO in preconditioning and cardioprotection is being increasingly recognised, and a variety of NO donors have been used (for summary, see reference 25), including sodium nitroprusside.[6,26] The data in Table II show that SNP pretreatment significantly improved cardiac output and work performance after exposure to 20 min global ischaemia. Interestingly, melatonin, when administered prior to and simultaneous with SNP, tended to improve rather than block functional recovery.

The beneficial cardioprotective actions of NO include activation of guanylyl cyclase, generation of cyclic guanosine monophosphate (cGMP), activation of protein kinase G (PKG) and opening of the mitochondrial KATP channels.[25] The results obtained in this study (Table II) suggest that the amounts of NO generated by SNP completely override the scavenging abilities of melatonin, or else that melatonin is not capable of scavenging this particular free radical. However, it has been shown that melatonin is capable of detoxifying NO.[27,28]

Melatonin has also been suggested to interact with a molecule derived from NO, possibly peroxynitrite. The results obtained in the present study could not show any evidence of NO scavenging by melatonin, since no deleterious effects on SNP-induced cardioprotection were observed. The interrelationship between melatonin and a NO donor in the perfused heart model needs to be further evaluated.

The results obtained with melatonin are in agreement with those obtained by other workers using free radical scavengers. For example, administration of ascorbic acid in pigs abolished the beneficial effects of preconditioning on infarct size,[29] while mercaptopropionyl-glycine diminished diazoxide-induced preconditioning.[30] As far as we know, the effect of melatonin on ischaemic preconditioning has previously been studied by one group only.[31] Using rabbits, melatonin (50 mg/kg) was found to have no effect on ischaemic preconditioning-induced reduction in infarct size, despite a reduction in lipid peroxidation and increase in superoxide dismutase (SOD) activity.

There are several reasons for the differences between our results and those obtained by Andreadou *et al*.[31] For example, animal species (rat vs rabbit), model (*in vitro* vs *in vivo*), preconditioning protocol (1×5, 3×5 min vs 2×5 min), period of ischaemia (20 vs 30 min), and severity of ischaemia (global vs coronary artery ligation) could all affect the outcome.

Finally, our results show that melatonin, at a concentration of 50 μM, is capable of abolishing the cardioprotective actions of a multi-cycle ischaemic preconditioning protocol, probably by virtue of its free radical-scavenging abilities. Melatonin may also exert its effects via its powerful anti-adrenergic actions,[32] which could attenuate the generation of cAMP, one of the triggers of ischaemic preconditioning.[33] In addition, we have previously shown that the cardioprotection induced by melatonin during reperfusion could be abolished by luzindole, a melatonin receptor blocker.[6] However, the role of the melatonin receptor in abolishing preconditioning remains to be established.

In summary, melatonin's actions on the perfused heart are dependent on the time of administration. If added during reperfusion only, melatonin is cardioprotective, while when administered before and during an ischaemic preconditioning protocol, it abolishes protection.

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References

1. Reiter RJ, Tan DX. Melatonin: a novel protective agent against oxidative injury of the ischemic/reperfused heart. *Cardiovasc Res* 2003; **58**: 10–19.
2. Reiter RJ, Tan DX, Mayo JC, Sainz RM, Leon J, Czarnocki Z. Melatonin as antioxidant: biochemical mechanisms and pathophysiological implications in humans. *Acta Biochim Pol* 2003; **50**: 1129–1146.
3. Lagneux C, Joyeux M, Demenge P, Ribuot C, Godin-Ribuot D. Protective effect of melatonin against ischemia-reperfusion injury in the isolated rat heart. *Life Sci 2000*; **66**: 503–509.
4. Sahna E, Parlakpinar H, Turkoz Y, Acet A. Protection of myocardium by melatonin against ischemia-reperfusion induced infarct size and oxidative changes. *Physiol Res* 2005; **54**: 491–495.
5. Dobsak P, Siegelova J, Eicher JC, Jancik J, Svacinova H, Vasku J, *et al*. Melatonin protects against ischemia-reperfusion injury and inhibits apoptosis in isolated working rat heart. *Pathophysiology* 2003; **9**: 179–187.
6. Lochner A, Genade S, Davids A, Ytrehus K, Moolman JA. Short- and long-term effects of melatonin on myocardial post-ischemic recovery. *J Pineal Res* 2006; **40**: 37–42.
7. Sahna E, Olmez E, Acet A Effects of physiological and pharmacological concentrations of melatonin on ischemia-reperfusion arrhythmias in rats: can the incidence of complete sudden cardiac death be reduced? *J Pineal Res* 2002; **32**: 194–198.
8. Sahna E, Acet A, Ozer MK, Olmez E. Myocardial ischemia-reperfusion in rats: reduction of infarct size by either supplemental physiological or pharmacological doses of melatonin. *J Pineal Res* 2002; **33**: 324–328.
9. Kaneko S, Okumura K, Numaguchi Y, Matsui H, Murase K, Mokuno S, et al. Melatonin scavenges hydroxyl radical and protects isolated rat hearts from ischemic reperfusion injury. *Life Sci* 2000; **67**: 101–112.
10. Lee YM, Chen HR, Hsiao G, Sheu R, Wang JJ, Yen MH. Protective effects of melatonin on myocardial ischemia/reperfusion injury in vivo. *J Pineal Res* 2002; **33**: 72–80.
11. Das DK, Maulik N. Preconditioning potentiates redox signalling and converts death signal into survival signal. *Arch Biochem Biophys* 2003; **420**: 305–311.
12. Chen W, Gabel S, Steenbergen C, Murphy E. A redox-based mechanism for cardioprotection induced by ischemic preconditioning in perfused rat heart. *Circ Res* 1995; **77**: 424–429.
13. Oldenburg O, Yang XM, Krieg T, Garlid KD, Cohen MV, Grover GJ, *et al*. P1075 opens mitochondrial KATP channels and generates reactive oxygen species resulting in cardioprotection of rabbit hearts. *J Mol Cell Cardiol* 2003; **35**: 1035–1043.
14. Pain T, Yang XM, Critz SD, Yue Y, Nakano A, Liu GS, *et al*. Opening of mitochondrial KATP channels triggers the preconditioned state by generating free radicals. *Circ Res* 2000: **87**: 460–466.
15. Oldenburg O, Qin Q, Krieg T, Yang XM, Phillips S, Critz SD, *et al*. Bradykinin induces mitochondrial ROS generation via NO, cGMP, PKG, and mito KATP channel opening and leads to cardioprotection. *Am J Physiol* 2004; **286**: H468–476.
16. Lochner A, Marais E, Genade S, Moolman JA. Nitric oxide: a trigger for classic preconditioning? *Am J Physiol* 2000; **279**: H2752–H2765.
17. US National Institute of Health. *Guide for the Care and Use of Laboratory Animals*. NIH publication no 85-23, revised 1985.
18. Kannengieser GJ, Opie LH, van der Werff TJ. Impaired cardiac work and oxygen uptake after reperfusion of regionally ischemic myocardium. *J Mol Cell Cardiol* 1979; **11**: 197–207.
19. Li GC, Vasquez JA, Gallagher KP, Lucchesi BR. Myocardial protection with preconditioning. *Circulation* 1990; **82**: 609–619.

20. Van Winkle DM, Thornton JD, Downey DM, Downey JM. The natural history of preconditioning: cardioprotection depends on duration of transient ischemia and time subsequent to ischemia. *Coron Art Dis* 1991; **2**: 613–619.
21. Sandhu R, Diaz RJ, Mao GD, Wilson GJ. Ischemic preconditioning. Differences in protection and susceptibility to blockade with single-cycle versus multicycle transient ischemia. *Circulation* 1997; **96**: 984–995.
22. Barbosa V, Sievers RE, Zaugg CE, Wolf CL. Preconditioning ischemia time determines the degree of glycogen depletion and infarct size reduction in rat hearts. *Am Heart J* 1996; **131**: 224–230.
23. Schulz R, Post H, Valhaus C, Heusch G. Ischemic preconditioning in pigs: a graded phenomenon. Its relation to adenosine and bradykinin. *Circulation* 1998; **98**: 1022–1029.
24. Lochner A, Genade S, Moolman JA. Ischemic preconditioning: infarct size is a more reliable endpoint than functional recovery. *Bas Res Cardiol* 2003; **98**: 337–345.
25. Cohen MV, Yang X-M, Downey JM. Nitric oxide is a preconditioning mimetic and cardioprotectant and is the basis of many available infarct-sparing strategies. *Cardiovasc Res* 2005; e-publication.
26. Draper NJ, Shah AM. Beneficial effects of a nitric oxide donor on recovery of contractile function following brief hypoxia in isolated rat heart. *J Mol Cell Cardiol* 1997; **29**: 1195–1205.
27. Mahal HS, Sharma HS, Mukherjee T. Anti-oxidant properties of melatonin: a pulse radiolysis study. *Free Rad Biol Med* 1999; **26**: 577–585.
28. Noda Y, Mori A, Liburty R, Packer L. Melatonin and its precursors scavenge nitric oxide. *J Pineal Res* 1999; **27**: 159–164.
29. Skyschally R, Schulz R, Gres P, Korth H, Heusch G. Attenuation of ischemic preconditioning in pigs by scavenging of free oxyradicals with ascorbic acid. *Am J Physiol* 2003; **284**: H698–H703.
30. Tanaka M, Fujiwara H, Yamasaki K, Sasayama S. Superoxide dismutase and mercaptopropionyl glycine attenuate infarct size limitation effect of ischemic preconditioning in the rabbit. *Cardiovasc Res* 1994; **28**: 980–986.
31. Andreadou I, Iliodromitis EK, Mikros E, Bofilis E, Zoga A, Constantinou M, *et al*. Melatonin does not prevent the protection of ischemic preconditioning in vivo despite its anti-oxidant effect against oxidative stress. *Free Rad Biol Med* 2004; **37**: 500–510.
32. Abete P, Bianco S, Calabrese C, Napoli C, Cacciatore F, Ferrara N, Rengo F. Effects of melatonin in isolated papillary muscle. *FEBS Lett* 1997; **412**: 79–85.
33. Lochner A, Genade S, Tromp E, Podzuweit T, Moolman JA. Ischemic preconditioning and the adrenergic pathway. *Circulation* 1999; **100**: 958–966.